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Identification, Cloning, Expression, and Characterization of the Gene for *Plasmodium knowlesi* Surface Protein Containing an Altered Thrombospondin Repeat Domain

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Proteins present on the surface of malaria parasites that participate in the process of invasion and adhesion to host cells are considered attractive vaccine targets. Aided by the availability of the partially completed genome sequence of the simian malaria parasite *Plasmodium knowlesi*, we have identified a 786-bp DNA sequence that encodes a 262-amino-acid-long protein, containing an altered version of the thrombospondin type I repeat domain (SPATR). Thrombospondin type 1 repeat domains participate in biologically diverse functions, such as cell attachment, mobility, proliferation, and extracellular protease activities. The SPATR from *P. knowlesi* (PkSPATR) shares 61% and 58% sequence identity with its *Plasmodium falciparum* and *Plasmodium yoelii* orthologs, respectively. By immunofluorescence analysis, we determined that PkSPATR is a multistage antigen that is expressed on the surface of *P. knowlesi* sporozoite and erythrocytic stage parasites. Recombinant PkSPATR produced in *Escherichia coli* binds to a human hepatoma cell line, HepG2, suggesting that PkSPATR is a parasite ligand that could be involved in sporozoite invasion of liver cells. Furthermore, recombinant PkSPATR reacted with pooled sera from *P. knowlesi*-infected rhesus monkeys, indicating that native PkSPATR is immunogenic during infection. Further efficacy evaluation studies in the *P. knowlesi*-rhesus monkey sporozoite challenge model will help to decide whether the SPATR molecule should be developed as a vaccine against human malarias.

An effective vaccine that reduces malaria-related mortality and morbidity would indeed alleviate the suffering of millions of people around the world (17). The onset and progression of malaria infection require a complex sequence of recognition, adhesion, and invasion events between the parasite and host cells. To accomplish this, the parasite expresses a multitude of proteins on its surface that serve as ligands that interact with the receptors present on the host cells. For example, circumsporozoite protein and thrombospondin-related anonymous protein (two sporozoite-stage proteins) participate in the binding of malaria sporozoites to liver cells (6, 30). In the erythrocytic-stage cycle, erythrocyte-binding antigen 175 is recognized as a merozoite ligand that binds to glycophorin A on erythrocytes to initiate the process of merozoite invasion (33). Likewise, the products of the *var* genes expressed on the surface of infected erythrocytes facilitate the adherence of mature forms of parasites to deep venules of endothelial cells and thus shield them from clearance by the host immune system.

The standard steps in preclinical vaccine development prior to phase I clinical trials in humans are antigen identification, its biochemical/biological characterization, and efficacy evaluation in animal models. For malaria, animal studies are generally performed using pertinent orthologs of *Plasmodium falciparum* (the most lethal form of human malaria) in the mouse (*Plasmodium yoelii*/*Plasmodium berghei*) and monkey (*Plasmodium knowlesi*) models. An analysis of preclinical efficacy studies performed over the last 20 years suggests that the high degree of protective efficacy observed in mice is generally difficult to achieve in humans, and studies on simian models may be more appropriate to predicate the vaccine potency in humans.

Infection with *P. knowlesi* in the Old World rhesus monkey *Macaca mulatta* is uniformly fatal and considered a reliable model to determine the efficacy of candidate vaccines against challenge with sporozoite or erythrocytic-stage parasites (31). While in nature the macaque monkey is its natural host, cross-species natural transmission of *P. knowlesi* to humans has been reported (21, 34), demonstrating the relevance of the rhesus-*P. knowlesi* model in evaluating the efficacy of malaria vaccines.

Recently, the genome sequences of *P. falciparum* and *P. yoelii* parasites have become available (5, 11), and efforts to generate partial or complete genome information for several other *Plasmodium* species are currently under way. The recent advances in bioinformatics have made it possible to assign putative biologic function to the majority of malarial antigens and, as a result, a large number of new antigens have become available for evaluation as vaccine candidates. However, in the absence of in vitro assays that could be used to predict vaccine efficacy, in vivo immunization-challenge studies remain the

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only credible method to identify novel protective antigens. This brings the question regarding how to predict antigens for further preclinical studies.

The complex life cycle of malaria parasites and stage-specific expression of the majority of malarial antigens present a unique challenge for vaccine development. Many malaria researchers believe that for a vaccine to be effective, it would be necessary to attack the parasite during multiple stages of its development. By this criterion, it is reasonable to assume that multistage, surface-expressed parasite proteins that are involved in the process of adhesion to and/or invasion of the host cells deserve special consideration as vaccine candidates.

Recently, we characterized a multistage *P. falciparum* secreted protein with an altered thrombospondin repeat (SPATR) (8) that is expressed at sporozoite, erythrocytic form, and gametocyte stages of the parasite. During the sporozoite stage, this protein is expressed on the cell surface and plays a role in the invasion of sporozoites into liver cells. Native *P. falciparum* SPATR is immunogenic, since immune sera from Ghanaian adults and from a volunteer who had been immunized with irradiated *P. falciparum* sporozoites recognized the recombinant *P. falciparum* SPATR expressed in transfected COS-7 cells (8).

The *P. yoelii* ortholog of this protein identified earlier (22) contains an altered thrombospondin repeat (TSR) domain, which is an ancient protein module that existed before the evolutionary separation of nematodes and vertebrates (18). In *Plasmodium* spp., the TSR domain is present in several surface proteins, and proteins encoding this domain have been implicated in diverse biologic functions, including parasite mobility, attachment to host cells, and host cell invasion (6, 19, 30). Two of the TSR domain-containing proteins, circumsporozoite protein and thrombospondin-related anonymous protein, are currently undergoing clinical trials as vaccine candidates (1, 24), suggesting that other *Plasmodium* proteins containing a TSR domain could also be potential vaccine targets.

In this report, we describe the identification, cloning, recombinant expression in *E. coli*, and biological characterization of a gene encoding a novel *P. knowlesi* protein with an altered TSR domain. Based on its homology to the *P. falciparum* SPATR protein, we named this protein PkSPATR. We believe that the availability of a well-characterized recombinant PkSPATR will expedite the preclinical efficacy determination of this biologically important molecule and help guide the decision as to whether this molecule should be further developed for clinical testing in humans.

MATERIALS AND METHODS

Parasites. *P. knowlesi* (Malaysian H strain) parasites were obtained from blood-stage infections of rhesus monkeys. Parasitized blood was passed through leukocyte reduction filters (Sepacell, Baxter, IL) to remove leukocytes. Infected red blood cells were used to isolate total RNA of *P. knowlesi* parasites using the High Pure RNA isolation kit (Roche Applied Science, Indianapolis, IN).

Reverse transcription, amplification, and cloning of DNA encoding PkSPATR. Total RNA from *P. knowlesi* was used for reverse transcription-PCR, using random hexamers and Superscript II RNase H^- reverse transcriptase (Invitrogen). The primer design was based on the BLAST search results in *P. knowlesi* database using published sequence of *P. falciparum SPATR* (GenBank accession number AE001404). The forward primer (5′ ATGAAAAAAAGTCGCTTTTTT 3′) and reverse primer (5′ ATTCTGATTGGTCGCTTCCAA 3′) were used to amplify the full-length 786-bp cDNA and it was cloned in the TOPO TA cloning vector (Invitrogen). The DNA sequence of PkSPATR was determined by automated sequencing.

Expression and purification of recombinant PkSPATR. For recombinant protein expression the forward primer (5′ CG GGATCC CCTTGAGTAAGAAA TTGTCCGGA 3′) and reverse primer (5′ CG GAATTC TTAATTCTGATTG GTCGCTTCCAA 3′) were used to amplify a 720-bp DNA fragment encoding the mature (Leu_{23} to Asn_{262}) PkSPATR protein. To facilitate cloning, BamHI and EcoRI restriction sites were introduced in the forward and reverse primers, respectively. The PCR-amplified fragment was cloned in-frame as a glutathione *S*-transferase (GST) fusion protein in pGEX-3X, a T7 promoter-based *E. coli* expression vector (Amersham Pharmacia Biotech), using BamHI and EcoRI sites.

For expression in *E. coli*, BL-21 cells were transformed with the *PkSPATR* plasmid and the expression was induced at an optical density at 600 nm of 1.0, with 1 mM isopropyl-1-thio-β-D-galactopyranoside, for 4 h. The total cell pellet was harvested by centrifugation at 4,000 × *g* for 20 min and resuspended in wash buffer (50 mM Tris, pH 7.5, 20 mM EDTA) containing lysozyme at a concentration of 0.5 mg/ml. Cell suspension was incubated at room temperature for 1 h with intermittent shaking. NaCl and Triton X-100 were added to obtain a final concentration of 0.5 M and 2.5%, respectively, and the suspension was further incubated at room temperature for 30 min, with vigorous shaking. This suspension was then centrifuged at 13,000 × *g* at 4°C, for 50 min, and the resultant pellet was resuspended in wash buffer containing 1% Triton X-100, using a Tissuemizer, and centrifuged at 13,000 × *g* for 50 min.

The pellet was washed repeatedly in wash buffer, without Triton X-100. After four washes, the pellet containing inclusion bodies was dissolved in 6 M guanidine hydrochloride and incubated for 2 h at room temperature followed by centrifugation at 50,000 × *g* at 4°C, for 30 min. Supernatant, containing denatured protein, was collected and the protein concentration was adjusted to 10 mg/ml with 6 M guanidine hydrochloride. Denatured protein was reduced by adding dithioerythritol, to a final concentration of 65 mM, and incubating at room temperature for 2 h. Protein was renatured by diluting 100-fold, in refolding buffer (100 mM Tris, pH 8.0, 0.5 M L-arginine-HCl, 2 mM EDTA, and 0.9 mM oxidized glutathione). After incubation at 10°C for 36 h, renatured material was dialyzed against 20 mM Tris, pH 8.0, and containing 100 mM urea. The renatured protein was loaded onto a Q-Sepharose column, and eluted by a salt gradient using a fast-protein liquid chromatography system. Relevant fractions were pooled and purified to homogeneity by gel filtration chromatography on the TSK-GEL G2000SW column. The purity of recombinant PkSPATR was determined on 4 to 20% polyacrylamide gels.

Generation of anti-PkSPATR antibodies. Anti-PkSPATR antibodies for immunofluorescence analysis were raised in mice by immunization with recombinant PkSPATR in Freund's adjuvant. Female 4- to 6-week-old CD1 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed, fed, and used in the experiments in accordance with the guidelines set forth in the National Institutes of Health manual *Guide for the Care and Use of Laboratory Animals*; 5 μg of recombinant PkSPATR in 100 μl phosphate-buffered saline was emulsified in 100 μl of complete Freund's adjuvant or incomplete Freund's adjuvant and was delivered by subcutaneous route at 3-week intervals. Nonheparinized whole blood was collected from the lateral tail vein of mice prior to immunization and 14 days after the last boost and serum samples were isolated.

Immunofluorescence assay. An indirect immunofluorescence assay was used to detect the expression of PkSPATR protein in sporozoite and erythrocytic stages of *P. knowlesi* parasites. Briefly, twofold diluted test sera were reacted with air-dried *P. knowlesi*-infected monkey erythrocytes. Antibodies were detected using fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (Southern Biotechnology, Birmingham, AL). Slides were mounted using VectaShield mounting medium for fluorescence (Vector Laboratories Inc., Burlingame, CA) and evaluated using a fluorescent microscope.

Hepatocyte binding assay. HepG2, a hepatoma human cell line, was grown in minimal essential medium, supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The assay was performed as described (27). Briefly, cells at a density of 100,000 cells per well were plated in 96-well plate 36 h before the experiment. The cells were fixed with 4% paraformaldehyde, followed by blocking with Tris-buffered saline containing 1% bovine serum albumin. Recombinant GST-PkSPATR or recombinant GST protein of *Schistosoma japonicum* expressed in *E. coli* at various concentrations was incubated with cells for 1 h followed by anti-PkSPATR antibody for 30 min and anti-mouse alkaline phosphatase-coupled conjugate for 30 min; 1 mM 4-methylumbelliferyl phosphate was used as the substrate, and fluorescence was measured in a fluorometer with excitation at 350 nm and emission at 460 nm.

Reactivity of *P. knowlesi*-infected *Macaca mulatta* serum to recombinant PkSPATR. Recombinant PkSPATR at 1 μg/ml concentration in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) was used to coat each well of a 96-well polyvinylchloride microtiter plate by overnight incubation at 4°C. The wells were blocked with 1% bovine serum albumin in 50 mM phosphate-buffered saline for 2 h at 37°C, washed three times with washing buffer (phosphate-buffered saline containing 0.05% Tween 20) and incubated for 60 min at 37°C with various dilutions of a pool of hyperimmune sera obtained from *M. mulatta* monkeys that had received multiple infections with *P. knowlesi* followed by drug cure. Unbound antibodies were washed out followed by an incubation with an alkaline phosphatase conjugate for 1 h. The plate was developed using 4-nitrophenylphosphate tablets (Sigma Chemical Co., St. Louis, MO) and the optical density at 412 nm was measured using an enzyme-linked immunosorbent assay reader.

RESULTS

Gene isolation and sequence analysis of PkSPATR. We searched the *P. knowlesi* genome sequence database (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/p_knowlesi), using the *P. falciparum* SPATR protein sequence as a TBLASTN query, and identified a contig (pkn907d01.q1c) that encoded two open reading frames showing reasonable sequence identity with *P. falciparum* SPATR. We have recently reported the characterization of SPATR, a multistage *P. falciparum* protein with an altered thrombospondin domain (8).

Using gene-specific primers identified on contig pkn907d01.qic, a 941-bp PkSPATR gene was PCR amplified using *P. knowlesi* genomic DNA prepared from asexual blood-stage parasites. Expression of a 786-bp PkSPATR transcript in *P. knowlesi* asexual-stage parasites was confirmed by reverse transcription-PCR. The genomic PCR and reverse transcription-PCR products were cloned in the TOPO-TA cloning vector (Invitrogen, San Diego, Calif.) and their nucleotide sequences were determined. The difference in the transcript size, as revealed by agarose gel electrophoresis (data not shown) and nucleotide sequencing, shows that PkSPATR is encoded by a two-exon gene separated by a short intron of 156 (Fig. 1a). Bioinformatic analysis of the nucleotide sequence predicted that the PkSPATR mRNA of 786 bases (GenBank accession number AY952327) translates into a 262-amino-acid-long polypeptide (Fig. 1b), with a predicted molecular mass of ~30.4 kDa (DNASTAR).

Analysis of the PkSPATR protein sequence using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) revealed that the first 21 amino acids encode a putative signal peptide with cleavage site between amino acids Lys_{21} and Glu_{22}. The carboxyl terminus of the protein has been predicted to have an altered thrombospondin repeat (TSR) domain that starts at Phe_{203} and ends at Asn_{260} (http://smart.embl-heidelberg.de/) (Fig. 1c). We compared the TSR domain of PkSPATR with that from several known and hypothetical *P. falciparum* proteins (Fig. 2). The TSR domain of PkSPATR has the conserved WSXW motif but differs from other TSR domains as it lacks the CSXTCG motif (where X is any amino acid). The predicted protein is rich in asparagine (6.87%) and lysine (8.02%).

The ScanProsite (12) algorithm reconfirmed the presence of the thrombospondin domain from amino acids 200 to 253 with the thrombospondin module WSPW (WSXW module, where X is any amino acid). This prediction algorithm also detected a putative N-glycosylation site [amino acids 31 to 34 (NSSL)] and a putative N-myristoylation site [amino acids 29 to 34 (GANSSL)] (Fig. 1c). Similar to *P. falciparum* and *P. yoelii*, PkSPATR is cysteine-rich and contained 12 cysteine residues. If the 262-amino-acid sequence of PkSPATR is divided in three equal parts of ~87 amino acids each, the N terminus (amino acids 1 to 87) has four cysteine residues, the central (amino acids 88 to 175) has two cysteine residues, and the C terminus (amino acids 176 to 262) has six cysteine residues.

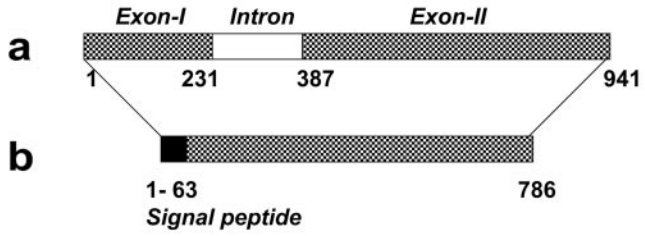
Cross-species sequence comparison of *Plasmodium* SPATR. The amino acid sequence alignment of SPATR protein orthologs from *P. knowlesi* (GenBank accession number AAX51302), *P. falciparum*, *P. vivax* (GenBank accession number AAX53168), and *P. yoelii* was done using ClustalW. SPATR protein sequences analyzed from four *Plasmodium* spp. share ~46% identity and ~57% sequence similarity based on charge, hydrophobicity, polarity, etc. The number and position of all the cysteine residues are conserved among these orthologs, indicating the conserved structure of this molecule within *Plasmodium* spp. Furthermore, the WSXW motif of TSR domain in SPATR is conserved in the *Plasmodium* genus with amino acid X being proline in PkSPATR and *P. vivax* SPATR, aspartic acid in *P. falciparum* SPATR, and asparagine in *P. yoelii* SPATR (Fig. 3a).

A neighbor-joining systematic tree of the SPATR molecule was constructed to study its phylogenetic relationship. PkSPATR and *P. vivax* SPATR cluster together and share ~83% sequence identity. *P. falciparum* SPATR and *P. yoelii* SPATR cluster independently. PkSPATR shares ~62% and 57% identity with *P. falciparum* SPATR and *P. yoelii* SPATR, respectively (Fig. 3b).

Expression of recombinant PkSPATR in *E. coli*. To produce PkSPATR as a recombinant protein in *E. coli*, we amplified the sequence coding for the mature protein (without signal sequence) by reverse transcription-PCR. A 720-bp (Leu_{23} to Asn_{262}) fragment obtained from the asexual stage parasite by reverse transcription-PCR was cloned as a BamHI/EcoRI-digested fragment in the pGEX-3X, a GST-based *E. coli* expression vector. The expression of recombinant PkSPATR in *E. coli* BL21 cells was induced with a 1 mM concentration of IPTG. The protein was overexpressed and its expression was readily detectable on a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. Further analysis revealed that PkSPATR was predominantly expressed as insoluble aggregates in the form of inclusion bodies.

In *E. coli*, the aggregation of PkSPATR could be due to the misfolding caused by incorrect disulfide linkage among the 12 cysteine residues. To purify the recombinant PkSPATR, inclusion bodies were isolated, denatured, and reduced, and the protein was subsequently renatured in vitro under redox conditions by diluting 100-fold, in refolding buffer (100 mM Tris, pH 8.0, 0.5 M L-arginine-HCl, 2 mM EDTA, and 0.9 mM oxidized glutathione). The renatured protein was subsequently purified to a high degree of homogeneity by column chromatography (Q-Sepharose column), and eluted by a salt gradient using a fast-protein liquid chromatography system. Since PkSPATR was expressed as a GST fusion, on purification, a single band of the expected molecular mass (~58 kDa) was visible on a Coomassie blue-stained gel (Data not shown).

Cellular localization of PkSPATR. To demonstrate the expression of PkSPATR on malaria parasites and to determine



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ATG AAA AAA AGT CGC TTT TTT TTC GTT TCC ATT TTT TTT TAT CTA TCT CAA GAG  54

S  S  L  E  L  S  K  K  L  S  G  A  N  S  S  L  T  E          36
AGC AGC CTG GAG TTG AGT AAG AAA TTG TCC GGA GCG AAC TCC TCC CTC ACA GAG 108

H  D  E  D  D  Y  E  H  A  H  K  R  P  R  T  W  S  A          54
CAT GAT GAA GAC GAT TAT GAG CAT GCA CAC AAG AGG CCC AGA ACG TGG TCG GCC 162

D  S  T  P  E  V  D  G  D  H  C  V  I  F  S  A  S  E          72
GAC TCG ACA CCG GAG GTA GAT GGC GAT ATG TGT GTG ATT TTC TCC GCC TCC GAG 216

G  D  P  T  K  C  W  C  P  R  G  Y  I  H  C  S  E  E          90
GGC GAC CCT ACC AAG TGC TGG TGC CCT AGA GGA TAC ATC ATG TGT AGC GAG GAG 270

D  V  R  D  V  Q  S  K  L  Y  E  I  K  D  L  K  Q  R         108
GAT GTG CGC GAT GTG CAG TCG AAG TTG TAC GAA ATT AAA GAC TTG AAG CAA CGG 324

N  E  V  T  P  P  W  H  K  R  L  C  D  N  S  E  E  I         126
AAC GAA GTG ACT CCC CCG TGG ATG AAG CGC CTG TGT GAT AAT TCA GAA GAA ATT 378

G  F  K  S  H  S  V  V  I  D  Y  E  L  A  V  L  C  N         144
GGA TTC AAA AGC ATG TCG GTT GTT ATA GAT TAT GAG TTG GCA GTT TTG TGC AAC 432

D  G  H  E  K  G  N  A  D  F  K  I  I  G  A  S  G  F         162
GAT GGA CAT GAG AAA GGC AAT GCA GAC TTT AAA ATA ATT GGT GCT TCA GGT TTC 486

V  P  N  E  K  I  I  Q  Q  K  E  R  D  I  T  Y  V  P         180
GTT CCG AAC GAA AAG ATC ATT CAA CAA AAA GAG AGG GAT ATC ACT TAT GTC CCA 540

R  K  C  T  V  N  N  F  Y  L  C  R  K  V  E  D  D  N         198
CGA AAA TGC ACC GTT AAT AAT TTT TAC TTG TGT CGA AAG GTG GAA GAC GAT AAT 594
                   W  S  X  W
V  G  C  Q  F  S  P  W  S  P  W  G  P  C  V  N  N  K         216
GTG GGT TGC CAG TTC AGC CCC TGG TCT CCG TGG GGT CCC TGC GTT AAT AAC AAA 648
                            TSP-1 Domain
Q  R  R  T  R  K  V  H  R  S  N  Q  N  N  E  N  F  C         234
CAG AGA AGA ACC AGG AAG GTG ATG CGC TCG AAC CAA AAT AAT GAA AAC TTT TGC 702

L  W  N  G  K  R  I  P  R  S  I  I  E  D  T  R  P  C         252
CTC TGG AAT GGC AAG CGC ATT CCG AGG AGC ATA ATA GAG GAT ACC CGC CCC TGC 756

K  I  A  L  E  A  T  N  Q  N                               262
AAG ATT GCA TTG GAA GCG ACC AAT CAG AAT                     786
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FIG. 1. Structural and compositional analysis of PkSPATR. (a) Structure of 941-bp PkSPATR gene showing two exons and the intron; (b) 786-base transcript of PkSPATR; (c) amino acid sequence of PkSPATR. The first 21 amino acids comprise the signal peptide (underlined). The thrombospondin domain (TSR), between amino acids 203 and 260, shown in shadow at the C terminus and the TSR module, WSXW, within the TSR domain is boxed. The putative N-glycosylation site at amino acids 31 to 34 (NSSL) is italicized and underlined, and a putative N-myristoylation site at amino acids 29–34 (GANSSL) is boxed. The 12 cysteine residues are underlined.

its cellular localization, we performed immunofluorescence analysis of *P. knowlesi* sporozoite and blood-stage parasites. To develop PkSPATR-specific antibodies for use in immunofluorescence assay, groups of five CD-1 outbred mice were immunized with 5 μg of purified recombinant PkSPATR in Freund's adjuvant (see Materials and Methods). In the immunofluorescence assay, a distinct reactivity of anti-PkSPATR antibodies was detected on the surface of *P. knowlesi* sporozoites (Fig. 4a) and intraerythrocytic stage parasites (Fig. 4b) showing the multistage expression of SPATR. The reactivity pattern observed in *P. knowlesi* sporozoites and intraerythrocytic-stage parasites was similar to that observed for *P. falciparum* parasites (8).

Biological function of PkSPATR in *P. knowlesi* sporozoites. Proteins containing one or more copies of TSR domains are crucial components of both the locomotive and invasion machineries (6, 25, 28, 36). The structural features of PkSPATR and its localization on the sporozoite surface suggested that this molecule might play a role in the sporozoite invasion of the

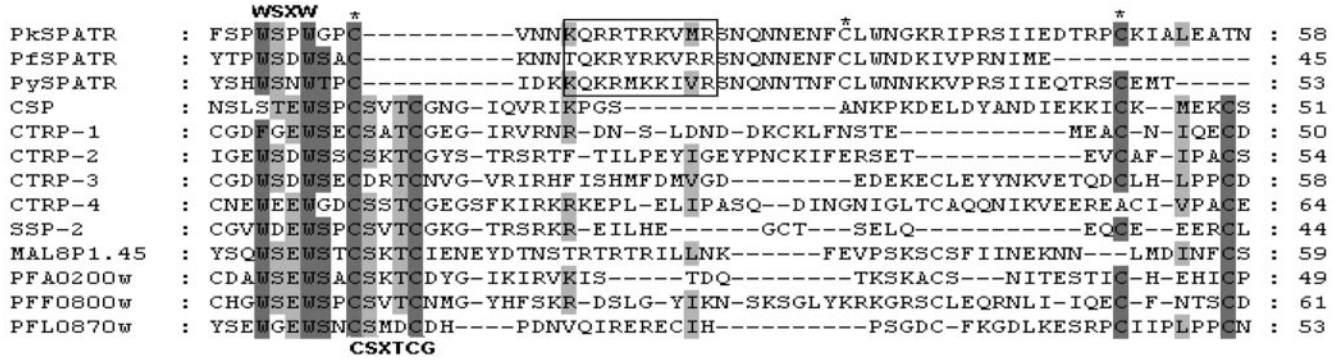

FIG. 2. Multiple amino acid sequence alignment of thrombospondin domains of various malarial proteins.

hepatocytes. To test this hypothesis, we investigated its ability to bind to HepG2, a human hepatocyte cell line, in an in vitro liver cell-binding assay. The binding of recombinant PkSPATR to HepG2 cells was determined at different protein concentrations. At a concentration of 2,000 nM, PkSPATR gave a fluorescence signal of 23,099, showing a high degree of binding to hepatocytes (Table 1). In comparison, at 2,000 nM, *E. coli* produced GST (fusion partner in recombinant PkSPATR), had a fluorescence signal of only 896 (Table 1), suggesting that the binding of PkSPATR to HepG2 cells was highly specific.

Recognition of PkSPATR by *P. knowlesi* infection-induced antibodies. Having established the biological role of PkSPATR in the liver cell-parasite interaction, we next wanted to determine whether PkSPATR was immunogenic during the course of infection with erythrocytic stage *P. knowlesi* parasites. Pooled sera from rhesus monkeys infected repeatedly with *P.*

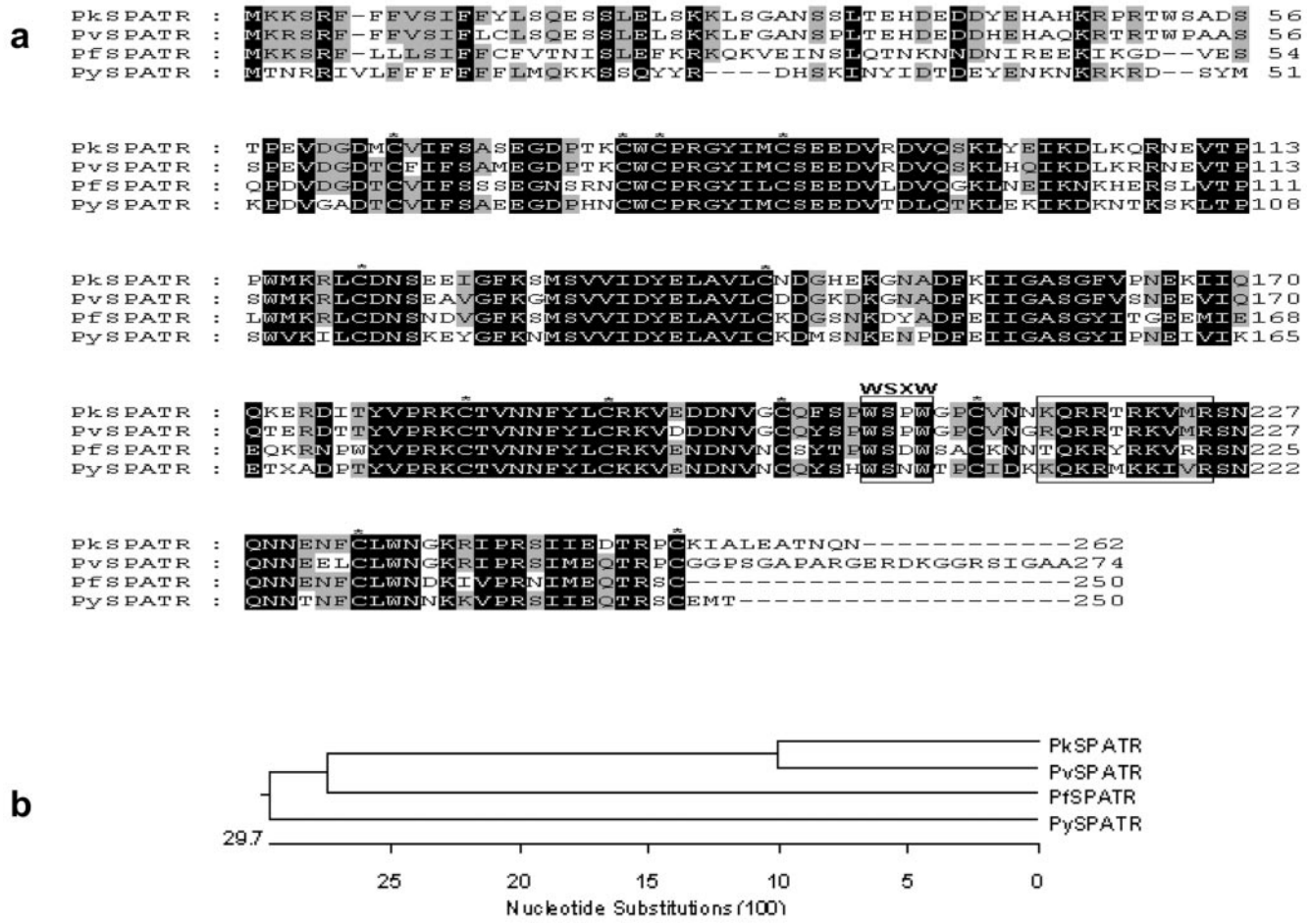

FIG. 3. (a) Multiple amino acid sequence alignment of SPATR proteins from four different *Plasmodium* species. (b) Phylogenetic relationship among SPATR proteins from *Plasmodium* spp.

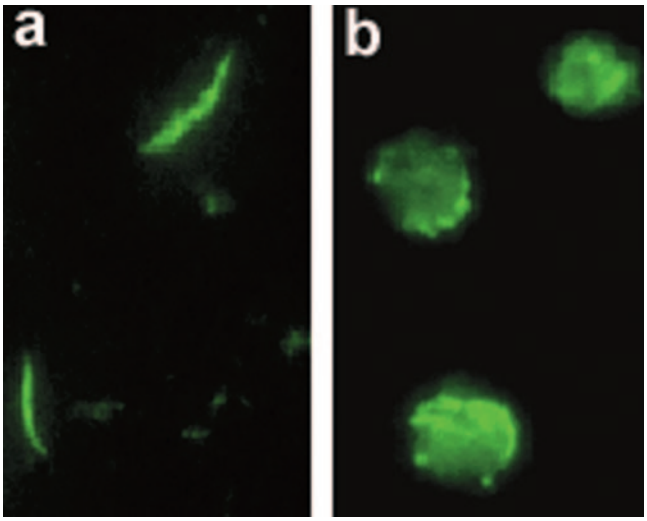

FIG. 4. Expression of PkSPATR during sporozoite and erythrocytic stages of *P. knowlesi* by immunofluorescence analysis. Sporozoite and blood-stage parasites were probed with anti-PkSPATR antibodies raised in mice using the recombinant protein. (a) Sporozoite; (b) erythrocytic stage.

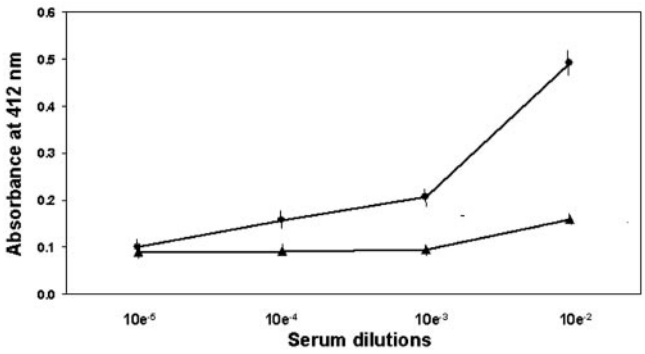

FIG. 5. Immune recognition of PkSPATR by hyperimmune sera of *P. knowlesi*-infected rhesus monkeys. Recombinant PkSPATR at a 1 μg/ml concentration was coated on to an enzyme-linked immunosorbent assay plate and various dilutions of hyperimmune serum were added, followed by the addition of anti-human immunoglobulin G conjugated to alkaline phosphatase. ▲, normal rhesus monkey serum; ●, pooled sera from immune rhesus monkeys generated by repeated *P. knowlesi* infections and drug cure.

knowlesi parasites reacted with recombinant PkSPATR in an enzyme-linked immunosorbent assay (Fig. 5). This showed that native PkSPATR in *P. knowlesi* parasites is immunogenic and induced antibodies that are recognized by epitopes present on recombinant PkSPATR.

DISCUSSION

The recent availability of the genome sequence of several *Plasmodium* species has paved the way for the identification of novel vaccine antigens. However, thus far, the genomics-based research has not led to the discovery of new vaccine candidates, and the majority of antigens under clinical development were identified at least a decade ago. Our ability to rapidly exploit the genome database for vaccine development is impeded by the lack of reliable criteria to select antigens for further preclinical development. One logical approach is to focus on molecules with assigned biologic functions such as invasion, sequestration, and their association with the progression of pathogenesis in the host.

In the present study, we report the gene cloning, recombinant expression, and biologic characterization of SPATR of *P. knowlesi* and its structural relationship with other *Plasmodium* species. The sequence analysis of PkSPATR revealed that it has a TSR domain at its carboxyl terminus. The TSR domain of PkSPATR has the WXSW motif but lacks the CSXTCG motif like its *P. falciparum* and *P. yoelii* orthologs. The other malarial proteins known to have thrombospondin domain are circumsporozoite protein, SSP2, and circumsporozoite protein/SSP2-related protein. These molecules predominantly play important roles in ookinete and sporozoite motility and host cell attachment and invasion (6, 25, 28, 36).

TABLE 1. Binding of PkSPATR to HepG2 cells[a]

Protein	Concn (nM)	Fluorescence (relative units) ± SE
PkSPATR	500	10,085 ± 1,443
	1,000	17,237 ± 1,003
	2,000	23,099 ± 1,285
GST alone	2,000	896 ± 38

[a] Cells were incubated with different concentrations of PkSPATR for 1 h, followed by the addition of anti-PkSPATR and anti-mouse IgG-alkaline phosphatase conjugate. The plate was developed by using 4-methylumbelliferyl phosphate as a substrate. The data represent the average of triplicate determinations.

In malaria parasites, the precise motif(s) of TSR involved in cell adhesion is not conclusively identified. The CSXTCG motif was first identified in circumsporozoite protein of *P. falciparum*, which has been shown to play a role as a cell adhesion motif for leukocytes (29). Other studies have identified the WSXW motif (amino-terminal to CSVTCG) as a heparin binding motif (13, 14) and the heparin binding affinity has been shown to be increased when a canonical BBXB motif (where B represents a basic residue, Arg, Lys, or His, and X represents any residue) was present adjacent to the WSXW motif. Other studies have found that the downstream basic amino acid residues are required for cell adhesion and not the CSVTCG motif. Another study showed that circumsporozoite protein binding to hepatocytes requires downstream basic amino acids but not the VTCG motif (10).

Given the complex life cycle of malaria parasites, many experts concur that a successful malaria vaccine may require the inclusion of several molecules expressed at various stages of the parasite development. The other alternative is to focus on biologically relevant antigens that are expressed during multiple stages of the parasites development. For example, SSP2/thrombospondin-related anonymous protein, one of the major surface proteins of the sporozoite stage, binds to both insect cells and human liver cells through a common glycosaminoglycan receptor (23). Recently, we have described *P. falciparum* SPATR, a multistage malaria protein having an alternative TSR domain that binds to human liver cells and plays a role in the invasion of sporozoite in the liver cells (8). *P. falciparum*

SPATR also binds to human erythrocytes and an anopheline mosquito larvae multipotent cell line (Chattopadhyay et al., unpublished data). Likewise, antibodies against apical membrane protein 1 can inhibit the invasion of sporozoites into liver cells and also block the merozoite invasion of human erythrocytes (2, 16, 32, 37). This suggests that the same malarial protein, expressed during different parasite stages, can recognize different receptors present on eukaryotic cells of both insect and mammalian origin.

Another multistage antigen called MB2, a 120-kDa protein, has been found to be present on the sporozoite surface and is imported into the nucleus of erythrocytic-stage parasites as a 66-kDa processed fragment (26). Although the receptor for MB2 protein is yet unknown, its differential processing and localizations at different stages of parasite development indicate that MB2 is capable of interacting with host receptors on both liver cells and erythrocytes. In this regard, we believe that SPATR, a multistage antigen present in several *Plasmodium* species, serves as a parasite ligand during the process of invasion into host cells.

Asparagine-rich motifs in proteins are targets of opsonizing antibodies that promote phagocytosis of parasites by immune cells (3, 4, 15). The abundance of the asparagine residue (6.87%) and the recognition of the recombinant PkSPATR by immune sera from multiple *P. knowlesi* infections and drug-cured rhesus monkeys in enzyme-linked immunosorbent assay indicate that PkSPATR could be a target of opsonizing antibodies.

In nature, cysteine is one of the least abundant amino acids but it is frequently found in the catalytic sites of the proteins. Malarial proteins rich in cysteine residues have often been implicated in parasite attachment and/or the invasion of the host cells. The primary examples of this observation are conserved cysteine-rich motif containing Duffy binding protein of *P. vivax* (9), Duffy-binding-like domain containing *P. falciparum* EMP1 (35), epidermal growth factor-like domain in $MSP1_{19}$ (20), and ecto-domain in AMA-1 (37). It has also been observed that specific position of the cysteine residue in a protein, like circumsporozoite protein, is important for maintenance of its biologic function (7, 27). PkSPATR has 12 cysteine residues, the majority of which are localized at the carboxyl and amino termini and less abundant in the central part of the protein. The abundance of cysteine residues and the ability to bind with liver cells strongly indicate that PkSPATR is a novel parasite ligand that plays an important role in attachment and/or invasion in host cells.

In summary, the data presented in this paper, and from our previous work, suggest that SPATR is expressed during multiple stages of parasite development and belongs to a family of malarial antigens that participate in the process of cell invasion and adhesion. Furthermore, the observation that *P. knowlesi* infection in rhesus monkeys induced anti-SPATR antibodies supports the argument that SPATR is a target of protective immune responses. We believe that the isolation, biological characterization, and production as a recombinant protein of SPATR from *P. knowlesi* will facilitate the preclinical efficacy evaluation of this biologically important molecule in a primate model.

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